Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713409232

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Jarrod E. Leland [a]; Donald E. Mullins [a]; Larry J. Vaughan [b]; Herman L. Warren [c]
[a] US Department of Agriculture, Agricultural Research Service, Southern Insect Management Research Unit, Stoneville, MS, USA [b] Office of International Research, Education, and Development, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA [c] Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

To cite this Article Leland, Jarrod E., Mullins, Donald E., Vaughan, Larry J. and Warren, Herman L.(2005) 'Effects of media composition on submerged culture spores of the entomopathogenic fungus, *Metarhizium anisopliae* var. *acridum*, Part 1: Comparison of cell wall characteristics and drying stability among three spore types', Biocontrol Science and Technology, 15: 4, 379 — 392
To link to this Article: DOI: 10.1080/09583150400016928
URL: http://dx.doi.org/10.1080/09583150400016928

Biocontrol Science and Technology, June 2005; 15(4): 379–392

Effects of media composition on submerged culture spores of the entomopathogenic fungus, *Metarhizium anisopliae* var. *acridum*, Part 1: Comparison of cell wall characteristics and drying stability among three spore types

JARROD E. LELAND[1], DONALD E. MULLINS[1], LARRY J. VAUGHAN[2], & HERMAN L. WARREN[3]

[1]*US Department of Agriculture, Agricultural Research Service, Southern Insect Management Research Unit, 141 Experiment Station Road, Stoneville, MS 38776, USA,* [2]*Office of International Research, Education, and Development, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA, and* [3]*Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA*

(Received 27 January 2004; returned 19 March 2004; accepted 16 September 2004)

Abstract

Metarhizium anisopliae var. *acridum* (IMI 330189) can produce at least three spore types *in vitro*; blastospores, submerged conidia, and aerial conidia, as defined by culturing conditions, sporogenesis, and spore morphology. This study compares morphological characteristics (dimensions and cell wall structure), chemical properties of cell wall surfaces (charge, hydrophobicity, and lectin binding), and performance (germination rate and drying stability) among these three spore types. Submerged conidia and aerial conidia both possessed thick, double-layered cell walls, with hydrophobic regions on their surfaces. However, in contrast to aerial conidia, submerged conidia have: (1) a greater affinity for the lectin concanavalin-A; (2) more anionic net surface charge; and (3) a less distinct outer rodlet layer. Blastospores were longer and more variable in length than both submerged conidia and aerial conidia, and had thinner single-layered cell walls that lacked an outer rodlet layer. Also, blastospores had a greater affinity than either conidia type for the lectin, wheat germ agglutinin. Blastaspores lacked hydrophobic regions on their surface, and had a lower anionic net surface charge than submerged conidia. In culture, blastospores germinated the fastest followed by submerged conidia, and then aerial conidia. Survival of submerged conidia and aerial conidia were similar after drying on silica gel, and was greater than that for blastospores. We provide corroborating information for differentiating spore types previously based on method of production, sporogenesis, and appearance of spores. These physical characteristics may have practical application for predicting spore-performance characteristics relevant to production and efficacy of mycoinsecticides.

Keywords: Metarhizium anisopliae, *conidia, blastospores, media, cell wall, mycoinsecticide, biocontrol*

Correspondence: Jarrod E. Leland, USDA-ARS-SIMRU, 141 Experiment Station Rd, Stoneville, MS 38776, USA. Tel: 1 662 686 3034. Fax: 1 662 686 5421. E-mail: jleland@ars.usda.gov

ISSN 0958-3157 print/ISSN 1360-0478 online
DOI: 10.1080/09583150400016928

Introduction

Entomopathogenic hyphomycete fungi produce aerial conidia *in vitro* from sporogenous hyphae known as phialides. Sporogenesis and morphology of this spore type are similar to aerial conidia produced *in vivo* from sporulating insect cadavers, a life stage adapted for survival outside the insect and infection (Hajek 1997). In nutrient-rich liquid media, entomopathogenic hyphomycete fungi typically produce thin-walled hyphal bodies known as blastospores *in vitro* by budding or septation (Adamek 1963; Jenkins & Goettel 1997). Sporogenesis and morphology of blastospores are more similar to hyphal bodies produced *in vivo* in the insect hemolymph, a life stage conducive for evasion of host immune system but not adapted for survival outside the host (Pendland et al. 1993; Hajek 1997). In liquid media, *M. anisopliae* can also produce asexual spores on phialides that have a green coloration, dimensions, and pip (fruit seed) shape similar to aerial conidia (Jenkins & Prior 1993). These spores we refer to as submerged conidia based on similarity to aerial conidia sporogenesis and morphology. If the stability of these spores is greater than blastospores, submerged conidia may combine the advantages of drying stability, shelf life, and field persistence with practical advantages of industrial-scale submerged liquid fermentation (Jenkins & Goettel 1997).

It is useful to describe morphological and cell wall surface characteristics of these three spore types to further define and discern them, and to predict performance characteristics relevant to mycoinsecticide development. Characteristics, such as hydrophobicity, surface carbohydrates, surface charge, stability, and pathogenicity, have been compared among these three spore types of the entomogenous hyphomycete, *Beauveria bassiana* (Bidochka et al. 1987, 1995; Thomas et al. 1987; Boucias et al. 1988; Hegedus et al. 1992; Pendland et al. 1993; Jeffs et al. 1999). In *B. bassiana*, blastospores are less hydrophobic than aerial conidia and submerged conidia (Hegedus et al. 1992; Bidochka et al. 1995; Jeffs 1999), they possessed relatively fewer surface carbohydrates (Hegedus et al. 1992; Pendland et al. 1993; Jeffs et al. 1999), are less stable in storage (Hegedus et al. 1992), but are the most pathogenic.

Submerged spores of *M. anisopliae* are generally less stable in storage than aerial conidia (Hegedus et al. 1992; Kleespies & Zimmerman 1994, 1998), although they may be stored under specific conditions to reduce metabolic activity or spray dried to improve shelf life (Kleespies & Zimmerman 1994; Stefan & Zimmerman 1998). In laboratory bioassays, *M. anisopliae* blastospores were more pathogenic than aerial conidia (Kleespies & Zimmerman 1994). In field trials, *M. anisopliae* submerged and aerial conidia had similar efficacy (Jenkins & Thomas 1996). Pathogenicity of *M. anisopliae* var. *acridum* submerged and aerial conidia are directly compared in laboratory bioassays in the accompanying paper (Leland et al. this issue).

The objectives of this study were to compare three spore types (blastospores, submerged conidia, and aerial conidia) of *M. anisopliae* var. *acridum* (IMI 330189) with respect to gross morphology (dimensions and cell wall structure), cell wall surface characteristics (hydrophobic microsites, surface charge, and lectin-binding), and performance characteristics (germination rate and drying stability). Characteristics described for spores in this study should not be regarded as definitive for all blastospores and submerged conidia, but rather as a starting point for comparison to spores having similar gross morphologies in future studies. This distinction is made for two reasons. First the terms blastospore and submerged

conidia infer specific characteristics of sporogenesis, which are not described in the present study. Rather the terms blastospore and submerged conidia will be used to simplify discussion, based on similarities in production media and gross spore morphologies between this study and that which has previously been reported for blastospores (Adamek 1963) and submerged conidia (Jenkins & Prior 1993). Second spores were produced under a narrow set of cultural conditions and the stability of the described characteristics was not determined in response to changing variables in culture conditions. This study is the first of a two part study. In the later study the impact of media osmolality on similar characteristics of conidia from a submerged conidia producing medium are investigated (Leland et al. this issue).

Materials and methods

Strains and culture conditions

All experiments were conducted with *M. anisopliae* var. *acridum* (IMI 330189), which was isolated from the grasshopper species *Ornithacris cavroisi* (Finot) (Orthoptera: Acrididae) in Niger by CAB International through the Lutte Biologique contre les Locustes et Sauteriaux (LUBILOSA) project, a project started in 1989 with the objective of developing biological means of controlling locusts and grasshoppers. Starter cultures were grown on Sabouraud dextrose agar (SDA) (Sigma Chemical Co.) at 24°C for 2 weeks in the dark. Conidia from these plates were harvested by scraping spores from the surface and suspending at 6×10^7 spores ml^{-1} in 0.05% Tween 80 (Sigma) as determined by hemacytometer. One-milliliter aliquots of these stock spore solutions were stored in 20% glycerol at −80°C and used as inocula for producing aerial conidia for all experiments. Aerial conidia used in experiments were grown on SDA in 100-mm Petri dishes for 2 weeks at 24°C in the dark.

Aerial conidia, produced in plate culture on SDA as described above, were scraped from the surface of the culture and suspended in 0.05% Tween 80. Spore concentrations were adjusted to 6×10^6 conidia ml^{-1} as determined by hemacytometer. To determine the viability of aerial conidia, subsamples (100 µl) of these inocula were spread on 100-mm Petri plates containing 2% malt agar (20 g l^{-1} malt extract (Sigma) and 15 g l^{-1} bacteriological agar (Sigma)) and incubated for 24 h at 24°C in the dark. Percent spore germination of the inocula was determined for a minimum of 200 spores at 400 × magnification (Goettel & Inglis 1997). To initiate liquid cultures, 50 ml of liquid media in each of four replicate 250-ml baffled Erlenmeyer flasks was inoculated with 1 ml of a 6×10^6 conidia/ml suspension for a total of 6×10^6 aerial conidia. Cultures were incubated under continuous agitation at 150 rpm and 24°C. To produce blastospores, Adamek's medium (Adamek 1963), without Tween 80 was used (30 g l^{-1} corn steep liquor (Sigma), 40 g l^{-1} glucose (Sigma), and 40 g l^{-1} yeast extract (Sigma)). To produce submerged conidia, a modification of Jenkins's medium (Jenkins & Prior 1993) was used (40 g l^{-1} waste brewer's yeast (Stroh's Batch, NPC-Inc, Eden, NC, USA); 40 g l^{-1} fructose (Sigma); and 50 g l^{-1} lecithin (l-α phosphatidylcholine, Type II-S from soybean, Sigma)). The pH of all media was adjusted to pH 7 with sterile 1 N, NaOH before autoclaving. Spores were harvested after 6 days of growth by adding 200 g of glycerol per liter to the fermentation product, then freezing at −80°C. Production of submerged conidia was repeated six times and production of blastospores was repeated three times. Gross morphology of each spore type appeared consistent among these when

observed at $\times 1000$ magnification under phase. The spore characteristics described below were determined for spores taken from four flasks of each media type and were determined once per source flask.

Spore dimensions and cell wall structure

The length and width of spores were measured for 30–45 spores of each type at $\times 1000$ magnification using an optical micrometer with phase microscopy. Spore cross-sections were observed using transmission electron microscopy to measure cell wall thickness and observe cell wall structure.

Spores were fixed for electron microscopy by suspending them in 0.05% Tween 80 and centrifuging at $2200 \times g$ to remove spores from suspension (Allied Fisher Scientific Microcentrifuge Model 235C). Spore pellets were re-suspended in modified Karnovsky's fixative (0.5% paraformaldehyde (Fisher Scientific) and 2.3% glutaraldehyde (Polysciences Inc.) in 0.1 M sodium cacodylate (Polysciences Inc.) at pH 7) and allowed to fix overnight at 4°C (Karnovski 1965). After fixation, samples were centrifuged ($2200 \times g$), re-suspended in 2% agar at 55°C, then centrifuged again. Cooled, solidified agar, was diced into approximately 1-mm^2 pieces. These pieces were rinsed with three changes (10 min each) of 0.1 M sodium cacodylate buffer. Entrapped spores were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h, followed by rinsing twice in 0.1 M sodium cacodylate for 10 min each. Dehydration was accomplished with ethanol by incubating for 10 min each in the following concentrations; 50, 70, 95 (twice), and 100% (twice); followed by propylene oxide (three times). The samples were then perfused with Epon 812 mixed 50:50 with propylene oxide for 12 h, followed by 6 h of incubation in 100% Epon 812 with continual rocking motion at 18 rpm (Thermolyne Speci-Mix). Samples were then embedded in fresh Epon 812 and cured for 48 h at 60°C. The embedded spores were sectioned using a microtome (Porter Blum MT2) and the sections were examined using a JEOL 100 CX-II transmission electron microscope. Photographs of 11–22 spores of each type were taken at $\times 19\,440$ magnification. Four measurements of cell wall thickness were taken where the longest and shortest axes divided the spore. Measurements of aerial conidia cell walls did not include the rodlet layer attached to the outer spore wall membrane.

Cell wall surface characteristics

Lectin binding. Three lectins were selected based on known binding to *M. anisopliae* hyphae (Pendland & Boucias 1986) or their utility in distinguishing spore types of *B. bassiana* (Hegedus et al. 1992). Concanavalin A (Con-A) from *Conavalia ensiformis* binds α-D-mannosyl and/or α-D-glucosyl residues. Wheat germ agglutinin (WGA) from *Triticum vulgaris* binds *N*-acetyl-β-D-glucosaminyl residues and oligimers. Phytohaemagglutinin (PHA-P) from *Phaseolus vulgaris* binds oligossaccharides. All lectins were conjugated with fluorescein isothiocyanate (FITC) (Sigma). Spores were washed five times by centrifuging at $2200 \times g$ (Fisher Scientific Model 59A microcentrifuge) and re-suspending in sterile distilled water. A 20-µl sample of each spore suspension (1×10^7 spores ml^{-1}) was placed on a glass microscope slide and left undisturbed for 5 min to allow spore adhesion to the glass surface. Spores were then fixed with two drops of cold acetone (-20°C). The slides were rinsed with 1 ml

of distilled water then flushed for 30 s with distilled water to remove non-adhering spores and lectin solutions. Spores adhering to the glass slides were then treated with 40 µl of lectin solution (200 µg protein ml^{-1} saline) in low-light conditions. The treated slides were rinsed with distilled water, wet mounted with cover slips, and observed immediately in low-light using an epifluorescence microscope (450–490 nm). Fluorescence was evaluated qualitatively for approximately 50 spores of each type.

Surface charge. Spore surface charge was evaluated on the basis of the number of spores binding to cationic beads (Fast Flow Q-sepharose, Sigma) and anionic beads (Fast Flow CM-sepharose, Sigma). Charged beads were diluted to 1/3 of the stock concentration with HPLC-grade water to produce 60–83 µeq ml^{-1} of Q-sepharose and 33–43 µeq ml^{-1} of CM-sepharose. Spore samples were washed five times by centrifuging at $2200 \times g$, re-suspending in sterile distilled water, and diluting to 2×10^7 spores ml^{-1}. Charged bead suspensions (200 µl) were combined with 300 µl of each spore suspension. These suspensions were vortexed for 30 s and then shaken (200 rpm) for 15 min. Samples were observed under 400 × magnification to determine the number of spores associated with individual beads. An initial field of view was selected blindly. The number of spores associated with each bead was determined for all of the beads in the field of view before moving to another one along a transect. The numbers of spores associated with at least 30 beads were determined for each spore type.

To further measure the charged binding of spores, chitin (anionic) and chitosan (cationic) flakes (Sigma) that had been pulverized with a dental amalgamator were also used to evaluate binding of each spore type. Suspensions of blastospores, submerged conidia, and aerial conidia were made as described above in the charged bead assay and 3 mg of either chitin or chitosan was added to 3 ml of each spore suspension. Samples were shaken for 1 h at 200 rpm at 24°C. The numbers of spores adhering to individual particles of chitin or chitosan (20 particles each) were determined as described for the charged beads.

Hydrophobicity. The hydrophobic microsphere assay (HMA) (Hazen & Hazen 1988) was used to compare the hydrophobicity of spores. A suspension of hydrophobic microspheres was made by combining 24 µl of blue-dyed low-sulfate latex microspheres (1 µm diameter, Polysciences, Inc.), with 2 ml of 0.05 M PUM buffer (26.8 g K_2HPO_4 H_2O, 7.3 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4$ $7H_2O$ adjusted to a pH of 7.2 with HCl). The latex microspheres and the PUM buffer were initially at 4°C and maintained in an ice bath while being combined. Each 100 µl spore suspension (1×10^7 spores ml^{-1}) was combined with 100 µl of the microsphere suspension in a 2-ml glass vial. The suspensions sat for 1–2 min at room temperature followed by vortexing for 30 s and then were immediately observed by bright field microscopy at × 1000 magnification. The numbers of beads associated with individual spores were counted for all spores within blindly selected fields of view until at least 50 spores had been observed.

Glassware used in cell wall surface characteristic experiments (lectin binding, surface charge, and hydrophobicity) was soaked in Contrad 70 detergent (Polysciences, Inc.) for 24 h, soaked in 1% HCl for 24 h, rinsed seven times with distilled water at 4°C, then heated at 170°C for 4 h.

Germination and drying stability

Rapid drying on silica gel was used to compare the drying stability of *M. anisopliae* var. *acridum* spores using the method of Cliquet and Jackson (1997). Three replicate samples of blastospores, submerged conidia, and aerial conidia were used at concentrations ranging from 3.4×10^8 to 6.4×10^8 spores ml^{-1}. Each spore type was subjected to three treatments. To address potential effects of media components and osmotic environment on spore survival during drying, spores were either left in their respective growth media (non-washed treatments), washed with and suspended in distilled water (water-washed treatments), or washed with and suspended in 5% polyethylene glycol (PEG) 600 (PEG-washed treatments). Aerial conidia did not receive the non-washed treatment because they were grown on solid media. PEG at 5% was iso-osomotic with the growth media (approximately 430 mOsm, Advanced Instruments Model 3 MO Plus Micro Osmometer). Spores were centrifuged at $2000 \times g$ and re-suspended in the appropriate washing medium three times to their original volume.

Prior to drying on silica gel, a sample of each spore suspension gel was diluted to approximately 1×10^6 spores ml^{-1} with sterile distilled water and spread on 2% malt agar + 0.001% benomyl. These plates were incubated at 24°C and germination was stopped with 20% formalin after 6 and 42 h of incubation. Percent germination was determined for at least 200 spores at $\times 400$ magnification. A spore was considered germinated if the germ tube was more than half the diameter of the spore. Percent spore germination following 6 h incubation was used to compare germination rates among spore types. Percent spore germination following 42 h incubation was used to compare viability among spore types. Benomyl was used in germination plates to prevent rapidly germinating spores from producing excess hyphae that would mask late germinating spores (Milner et al. 1991).

Non-indicating silica gel (Type II, Sigma) was mixed with deionized water for 5 min and then placed in a drying oven at 100°C for 24 h. This process was repeated until the silica gel had broken into particles of approximately 2 mm diameter. The silica gel particles (1 g) were placed into 15-ml glass screw cap vials. For subjecting spores to desiccation stress, spore aliquots (100 µl) from each spore washing treatment (non-washed, water-washed, PEG-washed) were added to three replicate vials containing silica gel. Vials were immediately vortexed for 10 s and then immediately rehydrated with 10 ml of distilled water. Resuspended spores were evaluated for percent germination after 42 h of incubation as described in the preceding paragraph.

Results

Spore dimensions and cell wall structure

Blastospores, submerged conidia and aerial conidia could be differentiated based on overall spore dimensions and thickness of their cell walls (Table I). Submerged conidia were similar in length to aerial conidia, but were narrower than both aerial conidia and blastospores (Table I; $\alpha = 0.05$; Tukeys HSD). Blastospores were significantly longer than both aerial conidia and submerged conidia (Table I; $\alpha = 0.05$; Tukeys HSD). Blastospores were shorter than those measured by Kassa et al. (2004) from Adamek's medium (6.5 vs. >8 µm), however in both studies they were the most variable spore type with regards to length.

Table I. Dimensions and cell wall thicknesses of *M. anisopliae* var. *acridum* IMI 330189 blastospores, submerged conidia, and aerial conidia.

Spore type	Length (μm)[1]	Width (μm)	Wall thickness (μm)
Blastospores	6.5±0.3 a[2]	4.2±0.1 a	0.10±0.01 b
Submerged conidia	5.8±0.1 b	3.2±0.1 b	0.15±0.001 a
Aerial conidia	5.7±0.2 b	4.2±0.1 a	0.17±0.02 a

[1]Values presented as mean±standard errors.
[2]Values followed by different letters are significantly different from values within the same column at the $\alpha = 0.05$ level (Proc GLM; Tukeys, HSD; SAS). $N = 35–40$ spores for spore dimensions and 11–22 spores for cell wall thickness measurements.

Blastospores were significantly thinner than submerged conidia and aerial conidia (Table I; $\alpha = 0.05$; Tukeys HSD). Zacharuk (1970) described the primary, secondary, and tertiary cell walls of *M. anisopliae* aerial conidia as having widths of about 600, 100–450, and about 150 Å, for a total width of 850–1200 Å, similar to the 1000 Å (0.1 μm) width described for aerial conidia in the present study (Table I). Stratification within the cell wall can be seen in both aerial conidia and submerged conidia (Figure 1). Attached to the outer cell walls of aerial and submerged conidia were electron-opaque layers which likely correspond to the rodlet layer containing hydrophobin proteins (Boucias et al. 1988; St. Leger et al. 1998). The rodlet layer surrounding submerged conidia was much thinner and less well defined than that observed in aerial conidia (Figure 1b). Parts of this layer could be observed detaching from the outer surface of the spore in sheets. This layer corresponds to what was described by Zacharuk (1970) as 'an amorphous, mucoid substance sparsely and irregularly attached to the spore wall membrane ... ', however the components of this layer on the *in vitro* conidia from the present study may differ from those described by Zacharuk (1970), which were attached to insect cuticle. The outermost (third) layer of the cell wall described by Zacharuk (1970) as a thin (about 150 Å), transparent layer, outlined by a thin dense, lipid-like coating could not be discerned in the present study. In addition, Zacharuk (1970) described the transition between the inner two layers of the cell wall as the primary wall blending into a more dense, longitudinally striate wall. Whereas, in the present study, both aerial conidia and submerged conidia possessed a thin, dense, electron-opaque layer that served as a boundary between the primary and secondary cell walls, rather than a blending of the walls. In aerial conidia, this electron-opaque boundary layer between the primary and secondary walls was continuous throughout the cell wall, whereas in submerged conidia it was present at one end of the cell wall and merged with the outer edge of the cell wall as the width of secondary wall tapered.

In both aerial conidia and submerged conidia there was a thickening of the outer cell wall layer at one end of the spore giving both spore types a characteristic 'pip' shape. This shape was described by Zacharuk (1970) as resulting from interconidial attachments as conidia are produced in chains with the pole of the conidia distal to the phialide being greater in diameter than the proximal pole, leading to an ovate shape.

Blastospores lacked the electron-opaque boundary layer that was observed between primary and secondary cell walls in aerial and submerged conidia and cell walls were relatively electron transparent and uniform in their transparency. Blastospores did not

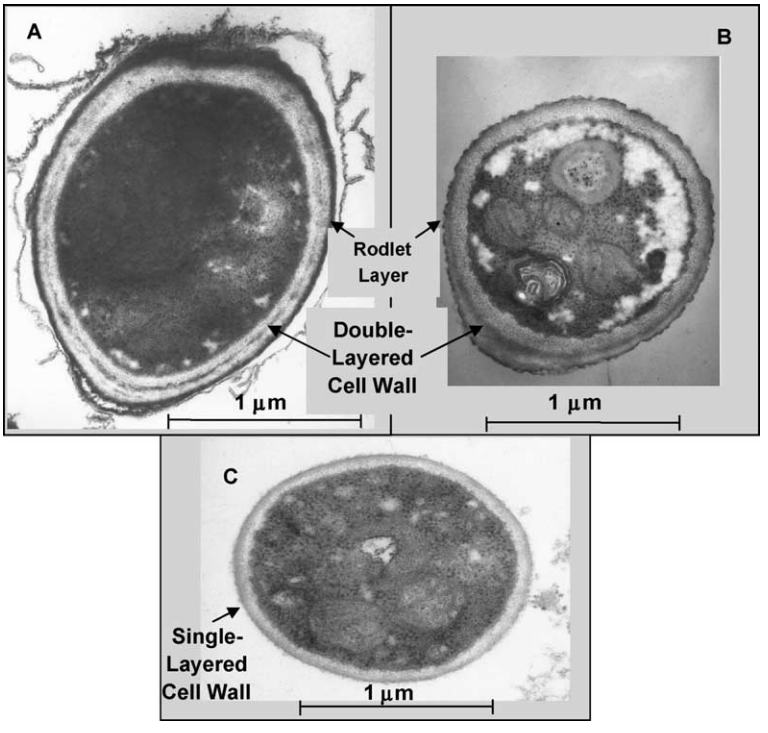

Figure 1. Transmission electron micrographs of typical (A) aerial conidium, (B) submerged conidium, and (C) blastospore. Since cross-sections may not bisect the spores' longitudinal axes, spore dimensions (length × width) in these micrographs do not necessarily correspond to spore dimensions measured under light microscopy in Table I.

have an electron-opaque layer attached to their outer cell wall indicating that they lacked a rodlet layer (Figure 1c).

Cell wall surface characteristics

Lectin binding. Blastospores, submerged conidia, and aerial conidia could be differentiated based on their lectin-binding characteristics (Table II). Concanavalin A (Con-A) bound very strongly to blastospores and submerged conidia, indicating the presence of exposed α-D-mannosyl and/or α-D-glucosyl residues. It bound weakly to aerial conidia indicating a limited amount of exposed residues. Phytohaemagglutinin P (PHA-P) did not bind to any of the spore types indicating an absence of exposed oligossacharides. Wheat germ agglutinin (WGA) bound strongly to blastospores indicating the presence of exposed *N*-acetyl-β-D-glucosaminyl residues. It bound very weakly to submerged conidia and aerial conidia indicating a limited amount of exposed residues. In many of the blastospores and submerged conidia, WGA appeared to bind preferentially to one end of the spores and bound very strongly to the few submerged conidia that were germinated, indicating a higher concentration of exposed *N*-acetyl-β-D-glucosaminyl residues in the germinating areas of the cell walls. A similar effect has been observed for germinating *Nomuraea rileyi* spores (Pendland & Boucias 1984) and was attributed to absence of the rodlet layer upon onset of germination exposing underlying carbohydrate residues.

Table II. Lectin-binding characteristics of *M. anisopliae* var. *acridum* (IMI 330189) blastospores, submerged conidia, and aerial conidia.

Spore type	Lectin		
	Con-A[1]	PHA-P	WGA
Blastospores	++++	0	+++
Submerged conidia	++++	0	+
Aerial conidia	++	0	+

[1]Con-A, concanavalin A (bind α-D-mannosyl and/or α-D-glucosyl residues); PHA-P, phytohaemagglutinin (binds oligosaccharides); WGA, wheat germ agglutinin (binds *N*-acetyl-β-D-glucosaminyl residues and oligimers).
[2]Values for fluorescence intensity are based on the following ranking (0) =no flourescence; (+) =very weak; (++) =weak; (+++) =moderate; (++++) =very strong fluorescence over the entire spore surface.

Surface charge. On the basis of number of spores associated with cationic (Q-Sepharose) and anionic (CM-sepharose) beads, submerged conidia were more anionic than either blastospores or aerial conidia. The latter two types did not demonstrate a strong attraction to either cationic or anionic beads (Table III; $\alpha = 0.05$; Tukeys HSD). There were significantly more aerial conidia associated with chitin and chitosan flakes than either submerged conidia or blastospores (Table III; $\alpha = 0.05$; Tukeys HSD). The numbers of blastospores, submerged conidia, and aerial conidia associated with chitin and chitosan flakes were highly variable, which may be due to the heterogeneous nature of the chitin and chitosan flakes (Table III). Spores were more often associated with darker more sclerotized fragments of chitin and chitosan than less sclerotized hyaline fragments of chitin and chitosan.

Hydrophobicity. A similar number of hydrophobic microspheres were observed in association with aerial conidia as with submerged conidia, indicating a presence of hydrophobic microsites on their cell wall surfaces; whereas, very few hydrophobic microspheres were associated with blastospores (Table III; $\alpha = 0.05$; Tukeys HSD).

Germination rate and drying stability

There were differences among the three spore types with regard to the percent of spores that germinated after 6 and 42 h incubation prior to drying over silica gel

Table III. Association of *M. anisopliae* var. *acridum* (IMI 330189) blastospores, submerged conidia, and aerial conidia with charged substrates or hydrophobic microspheres.

Spore type	Spores/ cationic bead[1]	Spores/ anionic bead	Spores/ chitinflake (−)	Spores/ chitosan flake (+)	Hydroph. spheres/spore
Blastospore	2.3±0.4 b[2]	1.0±0.2 a	1.0±0.5 b	0.5±0.3 b	0.2±0.1 b
Submerged conidia	12.9±1.4 a	1.7±0.3 a	0.2±0.1 b	1.1±0.3 b	1.3±0.2 a
Aerial conidia	1.5±0.3 b	1.8±0.4 a	4.7±1.7 a	6.0±2.8 a	1.5±0.2 a

[1]Values presented as means±standard errors.
[2]Values followed by different letters are significantly different from values within the same column at the $\alpha =$ 0.05 level (Proc Anova; Tukeys, HSD; SAS). $N = 30$ Beads for Q-sepharose and CM-sepharose 20 flakes for chitin and chitosan, and 50 spores for hydrophobic microsphere assay.

(Table IV). These values for percent spore germination prior to drying on silica gel are only presented from the non-washed treatments of each spore type because there was no significant effect by washing. The relative percentage of viable spores germinating following 6 h of incubation (RP6) are presented as percent of spores germinating at 6 h divided by the percent of spores germinating at 42 h, which is assumed to represent the population of viable spores. The RP6 was highest for blastospores followed by submerged conidia and then aerial conidia (Table IV; $\alpha = 0.05$; Kruskal–Wallis). The germination of blastospores following 42 h incubation was only 54%, which was significantly lower than submerged conidia and aerial conidia (Table IV; $\alpha = 0.05$; Kruskal–Wallis).

Drying on silica gel greatly reduced the viability of all spore types based on 42-h percent germination values (Table IV). Dried blastospores and submerged conidia survived significantly better if growth media was not washed from them (Table IV; $\alpha = 0.05$; Kruskal–Wallis). Spore survival for blastospores and submerged conidia washed in iso-osmotic PEG solution was similar to their counterparts washed in distilled water, suggesting that the protective effect of growth media on drying stability was not solely related to the osmolality of the growth media. Survival of submerged and aerial conidia were similar after drying when the two spore types were washed with distilled water or 5% PEG solution based on comparisons within each washing treatment (Table IV; $\alpha = 0.05$; Kruskal–Wallis). Survival of submerged conidia and aerial conidia after drying was consistently higher than that of blastospores when compared across the same spore-wash treatments (Table IV; $\alpha = 0.05$; Kruskal–Wallis).

Discussion

M. anisopliae var. *acridum* IMI 330189 produces at least three distinct spore types *in vitro*: aerial conidia, submerged conidia, and blastospores. These spore types can be differentiated on the bases of spore dimensions, cell wall thickness, cell wall surface characteristics, germination rate, and drying stability. The results of this study support

Table IV. Percent germination for *M. anisopliae* var. *acridum* (IMI 330189) blastospores, submerged conidia and aerial conidia before and after drying on silica gel.

	% Germination before drying		% Germination after drying (42-h incubation)		
Spore type	RP6[1]	Total germination (42-h) % Germ	With media[2]	Distilled water	PEG solution
Blastospores	55 ± 6 a	54 ± 1 a	20 ± 4 a[3], A[4]	2 ± 1 a, B	4 ± 1 a, B
Submerged Conidia	29 ± 3 b	95 ± 1 b	47 ± 4		
b, A	12 ± 2 b, B	9 ± 0 b, B			
Aerial Conidia	1 ± 1 c	94 ± 1 b	N/A	17 ± 4 b, B	12 ± 2 b, B

[1]The relative percent germination after 6 h (RP6) is the actual percent germination at 6 h divided by the percent germination of the same plates at 42 h × 100% (means ± standard errors).
[2]Spores were washed using three treatments before drying; media = left in growth media, distilled water = washed with distilled water, and PEG solution = washed with polyethylene glycol solution iso-osmostic with growth media.
[3]Values followed by different lower-case letters are significantly different within the same column ($\alpha = 0.05$; Kruskal–Wallis).
[4]Values followed by different capital letters are significantly different within the same row ($\alpha = 0.05$; Kruskal–Wallis).

the results of Jenkins and Prior (1993), which indicate production of submerged conidia by *M. anisopliae* var. *acridum* (IMI 330189) and isolates of *M. flavoviride* based on spore shape, pigmentation, and production on phialides, and further describe differences among these spore types. Although this study demonstrates several differences in characteristics among these spore types, limitations to interpretation of these results should be considered. Spore types were described as blastospores or submerged conidia based on similarities in gross spore morphology and selected media to those previously described for blastospores and submerged conidia. However, these terms are based on characteristics of sporogenesis, which were not described in this paper. Also, characteristics were described for spores grown under one set of conditions to produce each spore type. There is no evidence that the characteristics described would be consistent for spores that have similar gross morphology but are grown under different conditions. This study should be interpreted as a starting point for describing spores that appear morphologically similar to that which has been described as submerged conidia and blastospores. Considerably more work is needed to determine how these characteristics are affected by changing variables in culture conditions and describe sporogenesis for spores under these changing culture conditions. Therefore, results should not be viewed as definitive characteristics of blastospores or submerged conidia.

Aerial conidia and submerged conidia were both thick-walled relative to blastospores. The presence of thick cell walls along one terminus of the longitudinal axis lended to their characteristic 'pip' (pumpkin seed) shapes. The thick cell walls of aerial and submerged conidia relative to blastospores may provide greater desiccation tolerance to these spore types, which corresponds to desiccation data in the present study. Aerial conidia and to a lesser degree submerged conidia, both possessed an outer electron-opaque rodlet layer, which may contribute to desiccation resistance, water repellency, aerial dispersal, and adhesion to the insect cuticle (Boucias et al. 1988). Hydrophobins, a component of rodlet layers, were shown to be expressed by *M. anisopliae* almost equal at pH 5 and 8 (St. Leger et al. 1998) and they may play a role as sensors of hydrophobic surfaces to cue infection processes (St. Leger et al. 1989). Although the rodlet layer was less distinct in submerged conidia, it was nevertheless equally attractive to hydrophobic microspheres. Attraction of hydrophobic microspheres to the spore surface indicates the presence of hydrophobic sites on the surface of the spore but does not necessarily indicate that the entire spore will be hydrophobic or behave in a net hydrophobic manner (Jeffs et al. 1999). This has been shown for *B. bassiana* submerged conidia, which have hydrophobic microsites that attract hydrophobic microspheres but do not behave in a net hydrophobic manner in salt-mediated aggregation and sedimentation assays (SAS) (Jeffs et al. 1999). The authors suggested that hydrophobic microsites on submerged conidia may be masked by adjacent anionic sites when assaying the net spore hydrophobicity in the SAS assay, and rodlets may be arranged differently in submerged conidia than aerial conidia. A similar situation may be present in submerged and aerial conidia of *M. anisopliae* based on the appearance of the rodlet layer and the anionic charge of submerged conidia. If the net hydrophobicity of aerial conidia is greater than that of submerged conidia, this may also explain their greater attraction to chitin and chitosan and be most important in the initial phases of attraction to insect cuticle (Boucias et al. 1988). Although our assays indicated that aerial conidia had the greatest propensity for associating with chitin and chitosan, the binding of aerial conidia may not be

equally strong for both substrates. In our assays, the spore-substrate complexes were not washed after being combined. However, in assays that included a washing step, Boucias et al. (1988) found that aerial conidia bound to chitosan but not chitin (Boucias et al. 1988). It is surprising that *M. anisopliae* aerial conidia were shown by Boucias et al. (1988) to bind with cationic substrates (chitosan and DEAE-Bio-Gel beads), but were not as attracted to the cationic beads used in the present study.

Based on lectin binding characteristics, blastospores had higher concentrations of surface carbohydrates than aerial and submerged conidia. Similar lectin-binding characteristics have been reported for *M. anisopliae* var. *major* hyphae (Pendland & Boucias 1986) as we found for blastospores. However, our results differ from those observed for *B. bassiana* in which aerial conidia and submerged conidia generally had higher concentrations of exposed carbohydrate residues than blastospores (Hegedus et al. 1992; Jeffs et al. 1999). One reason for our results may be that the rodlet layer of aerial conidia masks the carbohydrates concentrated on their underlying cell walls. This explanation was proposed by Pendland and Boucias (1984) for *N. rileyi*. *Beauveria bassiana* blastospores produced *in vivo* lack surface carbohydrates, which may function in evasion of the host immune system (Pendland et al. 1993). If *M. anisopliae* blastospores produced *in vivo* similarly lack surface carbohydrates, they would differ in this characteristic from those produced *in vitro*, which possess relatively high concentrations of surface carbohydrates.

The germination rates (RP6 values) were highest for blastospores, followed by submerged conidia and then aerial conidia, which correspond to relative germination rates for *B. bassiana* blastospores, submerged conidia, and aerial conidia (Thomas et al. 1987). They also correspond to results indicating that *M. anisopliae* var. *anisopliae* blastospores are more infective than aerial conidia in laboratory bioassays (Kleespies & Zimmerman 1994).

Although blastospores germinate faster and, therefore, may be more infective to host insects, this benefit may be offset by the cost of their relative instability. One measure of spore stability is their survival following rapid drying (Cliquet & Jackson 1997). Leaving blastospores and submerged conidia in their growth media dramatically improved their survival after rapid drying on silica gel; an effect also observed by Cliquet and Jackson (1997) for *Paecilomyces fumosoroseous*. Similarly, additives may be used to protect submerged spores during spray drying to improve shelf life (Stephan & Zimmerman 1998). In the present study, the protective effect of the media does not appear to be an osmotic effect because spores suspended in PEG solution that is iso-osmostic to growth media did not survive drying as well as spores suspended in growth media. Low viability of blastospores prior to drying may have resulted from freeze–thaw damage or poor survival at -80°C. The same results occurred for *B. bassiana* blastospores (Hegedus et al. 1992), which further indicate the relative instability of blastospores. Survival rates following rapid drying were similar between submerged conidia and aerial conidia and were greater than that of blastospores, which may be attributed to their thicker conidial cell walls or protective effects of the rodlet layers (Boucias et al. 1988).

This study compares morphology, cell wall surface properties, and performance characteristics among blastospores, submerged conidia, and aerial conidia of *M. anisopliae* var. *acridum* (IMI 330189) to advance our understanding of these spore types and for application to mycoinsecticide development. Submerged conidia possess desired attributes of blastospores (relatively fast germination rates) and aerial

conidia (desiccation tolerance). The ability to produce a stabile spore type in submerged culture may have practical use in improving mycoinsecticide efficacy and efficiencies in spore production technology.

Acknowledgements

This research was funded by a USAID/Africa Bureau Project Grant No. AOT-G-00-97-00386-00 entitled *Development of Biopesticides for Grasshopper and Locust Control in Sub-Saharan Africa* managed by the Office of International Research, Education, and Development at Virginia Polytechnic Institute and State University. Initial cultures of *Metarhizium anisopliae* var. *acridium* (IMI 330189) were provided by CABI Biosciences and the LUBILOSA project. Technical assistance for transmission electron microscopy was provided by June Mullins.

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